Original Research Article

Antitumor effect and mechanism of action of polysaccharides extracted from *Polygonum perfoliatum* L whole plant in human lung carcinoma A549 cell line

Xi-xi Lai* and Yu-ping Li
Department of Respiratory Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325000, PR China

*For correspondence: **Email:** xxlaiwo@126.com; **Tel:** +86-0577-88669880*

Abstract

Purpose: To optimize the extraction conditions of polysaccharides from Polygonum perfoliatum L. (PSDP) and to evaluate their anti-tumor activities on A549 cell line.
Methods: Extraction of PSDP was optimized using Box-Behnken design (BBD). Three factors of response surface methodology (RSM) including extraction time, ratio of water to raw material and number of extractions were employed to optimize the yield of PSDP. The cytotoxic effect of PSDP on human lung carcinoma A549 cell line was evaluated in vivo, while its effects on expressions of caspase-3, caspase-9, Bcl-2 and Bax were determined by western blot assay.
Result: BBD was significant and applicable to PSDP extraction. Based on the contour plots, response surface plots and variance analysis, it predicted that the optimum conditions for PSDP extraction were: 1.58 h (extraction time); 30.18 mL/g (ratio of water to raw material); and 2.02 (number of extractions). PSDP had significant inhibitory effect on the growth of A549 cells in a concentration- and time-dependent manner (p < 0.05). After treatment with PSDP, caspase-3, caspase-9 and Bax were significantly up-regulated (p < 0.05), whereas Bcl-2 was down-regulated, all concentration-dependently.
Conclusion: RSM analysis is an appropriate method to optimize PSDP extraction. The results also indicate that PSDP has significant anti-tumor effect against A549 cells, most likely via inducing mitochondria-mediated apoptosis.

Keywords: Polygonum perfoliatum, Polysaccharides, Anti-tumor effect, Human lung carcinoma, Mitochondria-mediated apoptosis

INTRODUCTION

It is well-known that lung cancers still remain one of the leading causes of cancer related death all over the world, especially in men aged more than 40 years [1]. There are two main types of lung cancers namely non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for about 80 % of lung cancer patients [2]. NSCLC is one of the leading causes of cancer mortality, and the 5-year survival rate below 20 % [3]. Furthermore, although surgery,

radiotherapy and chemotherapy are used for the treatment of lung cancer, the treatment outcomes are usually unsatisfactory [4,5]. Therefore, it is important to explore newer and more effective approaches for the treatment of this disease.

The antitumor effects of Traditional Chinese Medicines (TCM) have aroused considerable interests recently. Some of the TCMs have been reported to possess unique advantages in treatment of various tumors [6,7]. *Polygonum perfoliatum* L. is a known TCM named

"Gangbangui" in Chinese. It is widely used in various places in China, especially in Guizhou province [8]. It is traditionally used for the treatments of cough, fever and edema, etc. [9]. *P. perfoliatum* has a variety of pharmacological properties such as anti-viral, anti-bacterial, anti-inflammatory and anti-tumor effects. [8,10-12]. However, not much is known about the active principles responsible for the observed anti-tumor effects of *P. perfoliatum*. Thus, it is necessary to investigate the active constituents of this plant, and relate them to its anti-tumor effect, with a view to proposing the likely mechanism(s) involved.

Our present study was designed to investigate the antitumor effects of polysaccharides extracted from *Polygonum perfoliatum* L. (PSDP) and the associated mechanisms, so as to provide a scientific basis for the clinical use of PSDP for treating lung cancer.

EXPERIMENTAL

Chemicals and reagents

Fetal bovine serum (FBS) and RPMI-1640 culture medium were purchased from Maichen Technology Co. (Beijing, China); 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA); Bcl-2, Bax, caspase-3 and caspase-9 monoclonal primary antibodies were obtained from Cusabio Biotech. (Wuhan, China). All the other chemicals used in the experiment were analytical grade.

Extraction of polysaccharides from PSDP

The whole plant of *P. perfoliatum* was obtained from Bozhou Traditional Chinese medicine market (Bozhou, China), and identified by the Department of Herbal Medicine in The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, PR China. A voucher specimen (no. ST20140115) was deposited at the herbarium of the hospital laboratory. The plant sample was dried, powdered and sieved through a standard mesh sieve (40 mesh). Then, the sieved powder was extracted by refluxing with distilled water at a designed ratio of water to raw material (20 mL/g, 30 mL/g, and 40 mL/g), extraction time (1, 1.5 and 2 h), and number of extractions (1, 2, and 3). The aqueous extract was filtered and concentrated under vacuum in a rotary evaporator (RE-2000A, Yarong Co., Shanghai, China). The concentrated solution was then precipitated with 75 % ethanol and left overnight at 4 °C. Thereafter the precipitate was

recovered by centrifugation at 5000 rpm for 15 min, and subsequently washed with anhydrous ethanol and acetone. The crude polysaccharide extract was dried and the extract yield was calculated using the formula:

Extraction yield (%) = $(w_0/w)100$ …………. (1)

where w_0 (g) is the weight of dried crude polysaccharides and w (g) is the weight of dried raw material.

Design of response surface methodology (RSM)

Based on the single factor analysis, a Box–Behnken design (BBD) was employed in the analytical process of RSM, in order to optimize the parameters (independent variables) of extraction namely: extraction time (A), ratio of water to raw material (B) and number of extraction (C). As a result, a total of 17 experiments were carried out in triplicate according to the BBD matrix (Table 1).

Cell culture

Human lung carcinoma A549 cell line was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were maintained in RPMI-1640 supplemented with 10 % fetal bovine serum and incubated at 37 °C in the presence of 5 % CO_2.

Evaluation of *in vitro* anti-tumor effect of PSDP

MTT assay was performed as previously described by Shao *et al* [13]. Human lung carcinoma A549 cells ($2.5 \times 10^4/0.2$ mL) were seeded into 96-well plates and treated with a series of concentrations of PSDP (20-800 μg/mL) for 48 h. After incubation, MTT solution (20 μL) was added to each well and incubated at 37 °C for 4 h. After that, the supernatant was discarded and 150 μL dimethylsulfoxide (DMSO) was added to each well. A microplate reader (Bio-Tek, USA) was used to measure the absorbance at 570 nm. Inhibition was computed according to formula below (Equation 2).

Inhibition (%) = {(Ac – At)/Ac)} ……………. (2)

where Ac and At are absorbance of control and treated samples, respectively.

Western blot analysis

Western blot assay was carried out to determine the protein expressions in A549 cell line

according to a previous study [14]. The A549 cells were suspended in RIPA buffer {150 mM NaCl, 0.1 % SDS, 1 % NP-40, 50 mM Tris-HCl (pH 7.4), 0.5 % sodium deoxycholate and protease inhibitors}, and the cell lysate was centrifuged at 20,000 g for 15 min at 4 °C. The total proteins were extracted, and equal quantities of proteins from each sample (40 µg) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. Proteins were subjected to immunoblotting analysis with respective primary and secondly antibodies (caspase-3, caspase-9, Bcl-2 and Bax). To normalize samples loading, β-actin was used. Subsequently, chemiluminescence reagents were added for visualization of the resultant protein bands.

Data analysis

Design Expert Version 7.0.0 software (State-Ease, USA) was used to analyze the experimental data of RSM. The fitness of the developed model and statistical significance of the regression coefficients were analyzed using Analysis of Variance (ANOVA). *P* < 0.05 was regarded as statistically significant.

RESULTS

Box-Behnken Design (BBD) output

Based on BBD design, 17 experiments were performed for different combinations of the extraction parameters to study the independent variables on the extraction yield of PSDP (Table 1). The experimental data was analyzed by multiple regression analysis, and the relationship between response variable and the test variables were shown according to be consistent with the following second-order polynomial equation:

$$Y = -13.931 + 16.702\,A + 0.428\,B + 0.122\,C - 0.002\,AB + 0.365\,AC + 0.016\,BC - 5.493\,A^2 - 7.558\times10^{-3}\,B^2 - 0.288\,C^2 \ldots\ldots\ldots (3)$$

ANOVA was used to analyze the adequacy and fitness of the model and identify the significant factors [15]. From the results in Table 2, the R^2 and R^2_{Adj} values of response surface model were 0.9890 and 0.9750, respectively. It showed goodness-of-fit between the experimental and the predicted values of the PSDP yield through the regression equation. The coefficient of variation (CV) value 3.10 clearly indicated low deviations between experimental and predicted values. In addition, F-value (70.22) and *p* value (< 0.0001) of the model indicated that the model was statistically significant. Lack of fit F-value (2.98) and the associated *p*-value (0.1592) were insignificant due to the relative pure error. These results showed that the model was significant and applicable.

Optimization of extraction conditions of PSDP

The contour plots and response surface were provided to illustrate graphically the relationship between the responses and the experimental variables. Each plot indicated the influence of two factors and the other factor was kept constant at its middle level. As shown in Figures 1a and 1b, when the number of extraction (C) was fixed at 2,

Table 1: BBD experimental design with the independent variables

Run	A (h)	B (mL/g)	C	Yield (%)
1	1	30	3	3.45
2	1.5	30	2	5.85
3	1.5	40	1	4.62
4	1	20	2	3.23
5	2	30	1	4.51
6	2	30	3	4.63
7	1	40	2	3.44
8	1.5	20	3	4.63
9	1	40	2	3.44
10	1.5	20	1	5.02
11	1.5	30	2	5.86
12	1.5	40	3	4.85
13	1.5	30	2	5.64
14	1	30	1	3.81
15	2	20	2	3.97
16	2	40	2	4.14
17	1.5	30	2	5.85

BBD = Box-Behnken Design

Table 2: Analysis of variance (ANOVA) of the BBD experiment

Source	Sum of Squares	df	Mean Square	F -Value	*P*-value
Model	13.37	9	1.49	70.22	< 0.0001
A	1.59	1	1.59	75.33	< 0.0001
B	5.000×10^{-3}	1	5.000×10^{-3}	0.24	0.6417
C	2.813×10^{-3}	1	2.813×10^{-3}	0.13	0.7261
AB	4.000×10^{-4}	1	4.000×10^{-4}	0.019	0.8945
AC	0.13	1	0.13	6.30	0.0404
BC	0.096	1	0.096	4.54	0.0705
A^2	7.94	1	7.94	375.44	< 0.0001
B^2	2.40	1	2.40	113.71	< 0.0001
C^2	0.35	1	0.35	16.54	0.0048
Residual	0.15	7	0.021	-	-
Lack of Fit	0.10	3	0.034	2.98	0.1592
Pure Error	0.09	4	0.022	-	-
Cor Total	13.51	16	-	-	-

Standard Deviation	Mean	C.V.%	Press	R^2	R^2_{Adj}	R^2_{Pred}	Adequate precision
0.15	4.69	3.10	1.71	0.9890	0.9750	0.8739	23.402

BBD = Box-Behnken Design

extraction time (A), ratio of water to raw material (B) showed a quadratic effect on the extraction yields of PSDP. However, the results in Table 2 showed that the interactions between variables A and B were not significant ($p > 0.05$). It has been reported that a circular contour plot implies that interactions between the variables are not significant [16]. Furthermore, a maximum extraction yield of PSDP was produced with increase of extraction time (A) and ratio of water to raw material (B). The same trend was exhibited in Figure 1e and Figure 1f. However, Figures 1c and 1d showed an elliptical contour plot. It indicated that the interactions between extraction time (A) and number of extraction (C) were significant ($p < 0.05$), which is consistent with the results in Table 2.

Based on contour plots, response surface plots and variance analysis, it could be predicted that the optimum conditions for PSDP extraction was as follows: extraction time was 1.58 h, ratio of water to the raw material was 30.18 mL/g and

number of extraction was 2.02. As a result, the predicted theoretical highest yield of PSDP was 5.86 %. To validate the adequacy of the developed model equations, modified conditions (extraction time 1.6 h, ratio of water to the raw material 30 mL/g and number of extraction 2) were used. The result showed average yield of PSDP was 5.77 %, which was well-matched with the predicted value, indicating that the RSM model was adequate.

Inhibitory effect of PSDP on human lung carcinoma A549 cells

The anti-tumor effect of PSDP on human lung carcinoma A549 cells was evaluated by the MTT assay. As shown in Figure 2, PSDP showed significant inhibitory effect on A549 cells in a concentration-dependent manner (20 - 800 µg/mL). It also had significant, time-dependent inhibitory effect on proliferation of A549 cells over a period of 72 h.
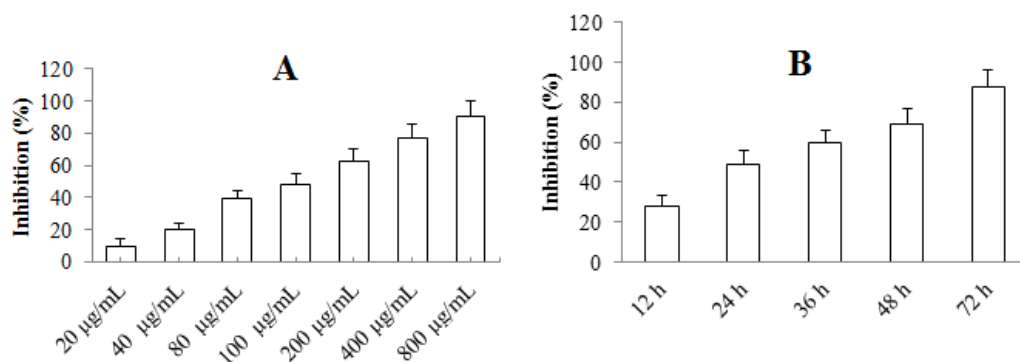

Figure 2: Inhibitory effect of PSDP on proliferation of human lung carcinoma A549 cells
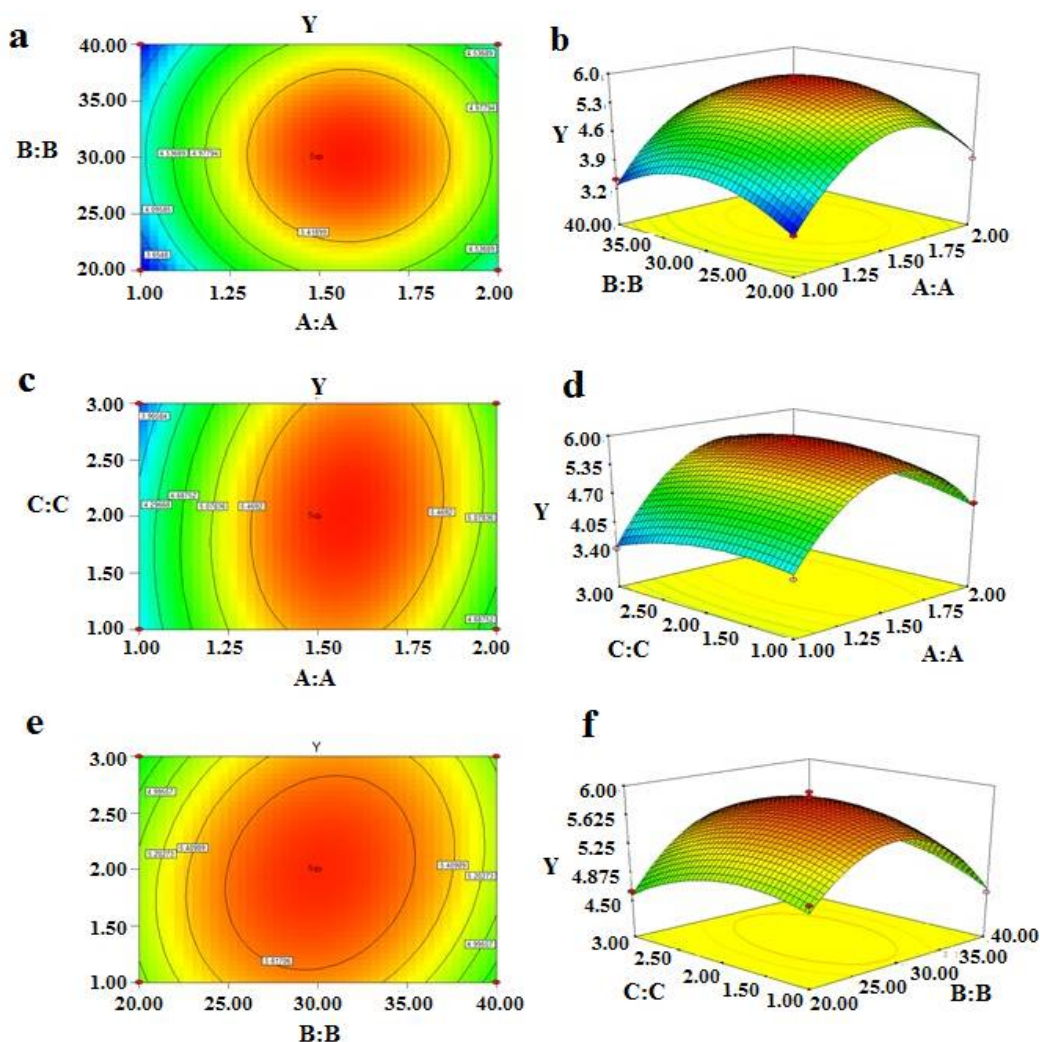
Figure 1: Contour plots and response surface plots showing effects of the variables on the yield of PSDP

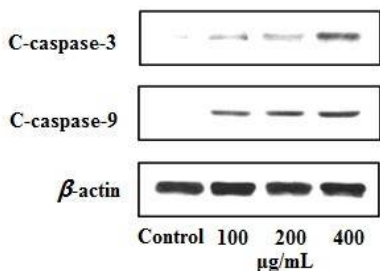**Regulatory effects of PSDP on c-caspase-3, c-caspase-9, Bax and Bcl-2**

Figure 3: Up-regulatory effects of PSDP on c-caspase-3 and c-caspase-9 in A549 cells

PSDP had significant and concentration-dependent up-regulatory effects on the expressions of caspase-3 and caspase-9 proteins, when compared with the control group (Figure 3). The expression of Bax was also significantly up-regulated by PSDP in a concentration-dependent manner relative to control (Figure 4). However, Bcl-2 expression was significantly down-regulated in a concentration-dependent fashion by the PSDP treatment.
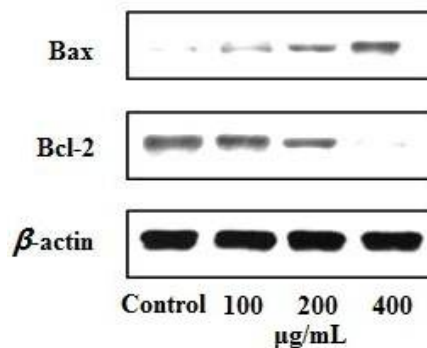

Figure 4: Regulatory effects of PSDP on Bax and Bcl-2 in A549 cells

DISCUSSION

RSM is an effective mathematical and statistical method for investigating and optimizing products or complex processes. In this method, quantitative data from an appropriate experimental design were used to determine and solve the multivariate equation simultaneously [17]. RSM can also provide abundant information, and it is economical because only a small number of experiments are needed for monitoring the interaction of independent variables based on the response [18].

BBD is a widely used design for the analysis of a response surface, and it has been used to extract biologically active compounds such as phenolic compounds, protein and polysaccharides from various sources [19]. In the present study, a BBD design was used to optimize the extraction of PSDP, and it was shown to be a suitable method for evaluating and optimizing the experimental variables such as ratio of water to raw material, extraction time, and number of extractions.

It has been established that in the apoptotic process, Bax gene, a pro-apoptotic member of Bcl-2 family, was re-distributed from the cytosol to mitochondria, with increase in membrane permeability, mitochondrial dysfunction and activation of caspases by induction of Bax gene. Mitochondrial cytochrome c was released and participated in the process, resulting in activation of caspase-9 (an initiator caspase), which in turn activates the executioner caspase, caspase-3 [14, 20]. This intrinsic activation pathway leads to induction of apoptosis in the cell.

In the present study, the expression of Bcl-2 was down-regulated, whereas Bax, caspase-3 and caspase-9 were increased after treatment with PSDP, in a concentration-dependent manner. These results indicated that the mechanism underlying the anti-tumor activity of PSDP on A549 cells is related to the induction of mitochondria-mediated apoptosis pathway.

CONCLUSION

RSM analysis is a suitable method for optimizing the extraction conditions of PSDP. PSDP has exerted significant anti-tumor effect against human lung carcinoma A549 cell line, most likely via induction of mitochondria-mediated apoptosis. The present study provides a scientific basis for the clinical use of PSDP for treating lung cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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